JOURNAL OF BACTERIOLOGY, Apr., 1966
Vol. 91, No. 4

Induction of Cellular Morphogenesis in *Myxococcus xanthus*

II. Macromolecular Synthesis and Mechanism of Inducer Action

WILLIAM SADLER AND MARTIN DWORKIN

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota

Received for publication 23 September 1965

ABSTRACT

SADLER, WILLIAM (University of Minnesota, Minneapolis), AND MARTIN DWORKIN. Induction of cellular morphogenesis in *Myxococcus xanthus*. II. Macromolecular synthesis and mechanism of inducer action. J. Bacteriol. **91**:1520–1525. 1966.—Net changes in ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein syntheses in cells of *Myxococcus xanthus* during induced, synchronous conversion to microcysts are described. The net synthesis of all three macromolecules was temporarily halted for a brief period during the initiation of shape change. Synthesis then resumed and leveled off when refractile microcysts began to appear. The conversion was completely sensitive, throughout the process, to low concentrations of chloramphenicol and actinomycin D. The uptake of amino acids and uracil was linear throughout the conversion, suggesting that the plateaus in rates of net synthesis of protein and RNA represented a period of rapid turnover. The most effective inducers of microcyst formation were fully saturated aliphatic compounds containing 2 to 4 carbon atoms and at least one primary or secondary alcohol group. Studies with labeled inducer indicated that the inducer need not be taken up by the cells to be effective, and probably interacts with some peripheral structure of the cell. The possibility that induction involves an alteration of a membrane-DNA complex is discussed.

During the normal developmental cycle in *Myxococcus xanthus*, vegetative cells are converted to spherical microcysts within fruiting bodies which are formed only on solid surfaces. It has been recently reported that this conversion can be effected rapidly, quantitatively, and synchronously in liquid medium by the addition of high concentrations of glycerol to suspensions of cells from the exponential phase of growth (1).

In the accompanying paper (2), a general description of this system is given. In this paper, we describe the macromolecular syntheses associated with microcyst formation together with some experiments designed to elucidate the mechanism of glycerol action.

MATERIALS AND METHODS

Vegetative cells of *M. xanthus* strain FB were grown and converted to microcysts by the glycerol technique as previously described (2).

For determinations of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, suspensions of washed cells were extracted with 0.25 N perchloric acid at 0 C for 30 min and then twice with 0.5 N perchloric acid at 70 C for 15 min. The combined 0.5 N perchloric acid extracts were analyzed for DNA by the diphenylamine method and for RNA by the orcinol method. The pellet remaining after the perchloric acid extraction was dissolved in 1 N NaOH at 90 C for 30 min and analyzed for protein by the method of Lowry et al. (4).

For the measurement of incorporation of radioactive compounds, these compounds were added to cell suspensions, and 0.1-ml samples were serially withdrawn and passed through Millipore filters. The cells on the filter were washed with 10 ml of cold 1% Casitone-0.01 M $MgCl_2$ medium. The filters were dried, and their radioactivity was determined in a Packard liquid scintillation spectrometer. Incorporation of C^{14}-amino acids and C^{14}-uracil into trichloroacetic acid-insoluble material was estimated by a modification of the filter-paper disc technique of Mans and Novelli (5).

RESULTS

Macromolecular syntheses during microcyst formation. Figure 1 shows the changes in the content of RNA, DNA, and protein of cells of *M. xanthus* during their conversion to microcysts after the addition of 0.5 M glycerol. RNA increased at a linear rate for the first 30 min, i.e., un-

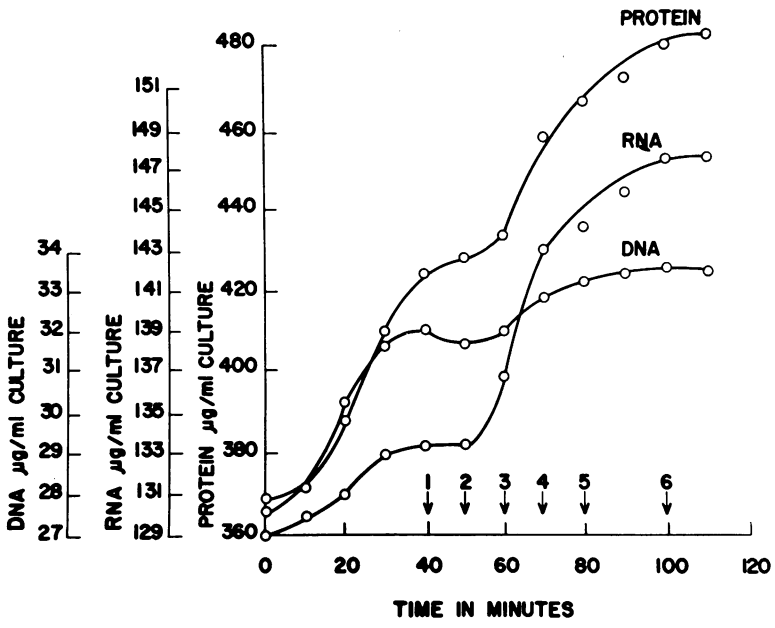

FIG. 1. *Synthesis of DNA, RNA, and protein during microcyst formation. Numbered arrows refer to morphological stages as follows: (1) vegetative rods, (2) rods beginning to shorten, (3) short rods, few ovoids, (4) ovoids and nonrefractile spheres, (5) nonrefractile spheres, (6) spheres beginning to acquire refractility.*

til about 20 min before the cells began to shorten. During the next 20 min, there was practically no change in RNA content. This was followed by a further increase as the cells began shortening and underwent the sequence of morphological changes culminating in refractile spheres. The RNA content leveled off at 100 min and showed a total increase of 20% during the conversion of the vegetative rod to a microcyst.

DNA and protein contents also increased during the conversion and in a manner rather similar to that of RNA. The decrease in the rate of protein synthesis seemed to lag about 10 min behind the decreases in the rates of RNA and DNA syntheses. For DNA and protein, the increases were 25 and 35%, respectively. The DNA-protein and RNA-protein ratios were essentially constant during the conversion. Phenethyl alcohol will also induce microcyst formation, albeit at a lower rate than glycerol (see *Other inducers of microcyst formation*). Under these conditions, the rates of RNA, DNA, and protein syntheses show the same stepwise pattern as when glycerol is used as the inducer. Furthermore, the changes in rates of net synthesis show the same relationship to morphological changes even though the conversion period is extended to about 12 hr.

Effect of inhibitors of protein and RNA synthesis. Chloramphenicol, added at a concentration of 0.5 μg/ml, produced a noticeable decrease in the rate of microcyst conversion. With 2.0 μg/ml, the time for the first appearance of cell shortening was increased from the usual 35 min to 90 min, and the subsequent conversion rate was very low. With higher concentrations of chloramphenicol, total inhibition of the conversion process was obtained.

To examine the chloramphenicol sensitivity of the various stages of the process, the inhibitor (5.0 μg/ml) was added at serial times after the addition of glycerol. When added between 0 and 20 min, chloramphenicol totally inhibited all morphological change and also the associated decrease in absorbancy. If cells were already half-shortened (about 50 min), they proceeded to make ovoids but went no further. When inhibitor was added at the ovoid stage, these cells very slowly became spherical but never became refractile, and the usual rise in absorbancy at this stage was not obtained. Finally, if the inhibitor was added when cells had become nonrefractile spheres, then these cells did subsequently acquire refractility but at a much reduced rate.

Actinomycin D added at time zero at a concentration of 0.1 μg/ml produced a considerable decrease in the conversion rate, and at 2.0 μg/ml inhibited all morphological change. When actinomycin D was added at serial times after the addition of glycerol, similar effects to those just reported for chloramphenicol were obtained; i.e.,

shortly after the addition of the inhibitor, microcyst development was either totally arrested or proceeded at a greatly reduced rate.

Incorporation of C^{14}-amino acids and C^{14}-uracil during microcyst formation. The rate of incorporation of C^{14}-amino acids into trichloroacetic acid-insoluble material by cell suspensions prepared in the usual way was found to be linear over the first 20 min (Fig. 2). Essentially similar results were observed either with C^{14}-leucine alone or with C^{14}-algal protein hydrolysate. This rate of incorporation showed no change upon the addition of glycerol and the subsequent conversion of cells to microcysts, at least up to the ovoid stage. Similarly, no change in the rate of incorporation of C^{14}-uracil into trichloroacetic acid-insoluble material was observed during the same period.

Other inducers of microcyst formation. As one approach to the problem of the mechanism of inducer action in this system, an attempt was made to find compounds other than glycerol which would also induce microcyst formation. After unsuccessfully trying a large variety of unrelated compounds it was found that phenethyl alcohol could effect the conversion, although only over a very narrow concentration range and at a considerably lower rate than glycerol. As the only obvious chemical similarity between phenethyl alcohol and glycerol was the possession of a primary alcohol group, a variety of alcohols were assayed for their ability to induce microcyst formation. These alcohols could be divided into four categories (Table 1): (i) those which induced microcyst formation, and at about the same rate as glycerol; (ii) those which induced microcyst formation, but at a much slower rate—usually requiring 4 hr or more; (iii) a group which caused the cells to shorten as far as the ovoid stage, but

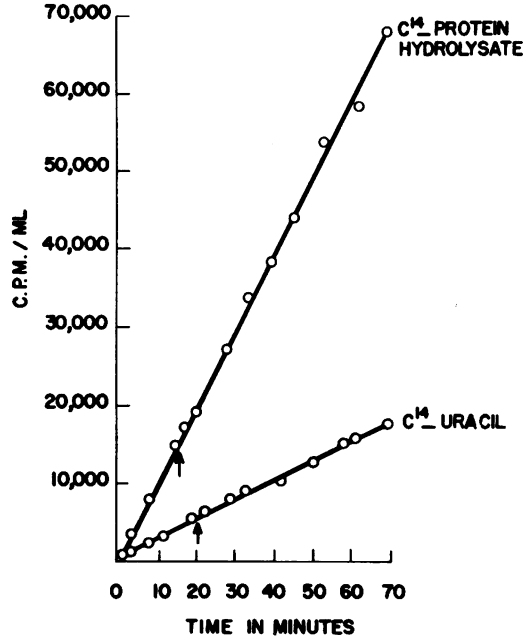

FIG. 2. *Incorporation of C^{14}-algal protein hydrolysate and C^{14}-uracil into trichloroacetic acid-insoluble material during microcyst formation. C^{14}-algal protein hydrolysate, 1.0 μc (specific activity 0.5 μc/0.278 μg), was added to 5.0 ml of cell suspension containing 7.0 μg/ml (dry weight) and aerated through a Pasteur pipette. Uracil-2-C^{14}, 0.5 μc (specific activity 22.9 μc/μmole), was added to a similar suspension. Samples (0.1 ml) were serially removed and the radioactivity in the trichloroacetic acid-insoluble fraction was determined by a modification of the method of Mans and Novelli (5). Glycerol to a final concentration of 0.5 M was added at times indicated by the arrows.*

TABLE 1. *Effect of various alcohols on microcyst production*

Effect	Alcohol	Concn
		M
Microcysts produced at same rate as with glycerol	*n*-Propanol	0.25–0.4
	Iso-propanol	0.25–0.4
	Ethylene glycol	0.5
	1,2-Propanediol	0.5
	1,3-Propanediol	0.5
	1,3-Butanediol	0.1–0.25
	1,4-Butanediol	0.1–0.25
	1,2,4-Butanetriol	0.1–0.25
	D-Threitol	0.5
	Erythritol	0.5
Microcysts produced slower than with glycerol	*n*-Butanol	0.1
	Iso-butanol	0.1
	Iso-amyl alcohol	0.025
	Cyclohexanemethanol	0.01
	Phenethyl alcohol	0.017
	Phenyl-1,2-ethanediol	0.05
	D-Ribose	0.3
	D-Xylose	0.3
Ovoids only produced	Ethyl alcohol	0.5–1.0
	Benzyl alcohol	0.1–0.025
	Cyclohexanol	0.05
No effect	Methanol	0.05–5.0
	t-Butanol	0.1
	n-Octanol	0.005
	Dihydroxyacetone	0.5
	2-Propyn-1-ol	0.05–0.5
	Allyl alcohol	0.05–0.5
	Glycidol	0.05–0.5

which never permitted complete microcyst formation; and (iv) those which had no detectable effect. The finding of a number of different compounds capable of inducing microcyst formation raised the question of whether the mode of action of these compounds is the same as that of glycerol. To date, only erythritol has been examined in some detail, and the evidence suggests that erythritol and glycerol may have the same mode of action. Thus, in terms of effective concentration range, requirement for continuous presence, antibiotic sensitivity, and the sequence of morphological and absorbancy changes, microcyst induction with erythritol was identical to that with glycerol. Furthermore, when cells were converted as far as the ovoid stage with glycerol, and then glycerol was removed and erythritol was added, these cells proceeded to make microcysts and to do so at the same rate as control suspensions. Similarly, the addition of glycerol to erythritol-induced ovoids resulted in complete microcyst formation. Finally, whereas neither 0.2 M glycerol nor 0.2 M erythritol alone permitted microcyst conversion, when added together, they were capable of doing so.

Incorporation and metabolism of inducers. The ability of cell suspensions of *M. xanthus* to incorporate some of these inducers of microcyst formation was next examined. It was found first that glycerol-*1,3*-C^{14} was incorporated by cell suspensions at a linear rate. The distribution of the incorporated label among the major cell fractions indicated that glycerol was apparently utilized as a general carbon source, with most of the label appearing in the ethyl alcohol- and hot trichloroacetic acid-soluble fractions (33 and 50%, respectively). When the initial glycerol concentration was 0.5 M, the amount of glycerol incorporated by cells during their conversion to microcysts was very small; thus, in one experiment the rate of incorporation by a cell suspension (10 mg/ml, dry weight) was 0.2 μmole per ml per hr.

When some of the other inducers listed in Table 1 were examined, their rates of incorporation were found to be much lower again. Thus, both C^{14}-erythritol and isopropanol-*1*-C^{14} were taken up by cell suspensions at barely detectable rates, even in the complete absence of corresponding carrier. Finally, with C^{14}-ethylene glycol, which is an excellent inducer, no incorporation of any label could be detected either by counting cells on Millipore filters or by adding small amounts of label and looking for disappearance of the label from the supernatant liquid.

Reversibility of lag period. One of the interesting features of the activity of the inducer in this system is the requirement for its continuous presence during almost the entire period of the morphogenetic change (2). If the inducer is withdrawn during this period, the sequence of morphological changes is reversed. To gain insight into the events initiated by the inducer, it was of interest to examine the question of reversibility during the lag period. This period of approximately 30 min between the addition of the inducer and the first appearance of morphological change presumably represents the time for a series of metabolic events to occur, culminating in the initiation of cell shortening.

In these studies, cells were exposed to glycerol for most of the lag period, then glycerol was withdrawn, and the cells were further incubated in its absence. Glycerol was then added back, and it was determined whether cells had to repeat the lag period or whether they proceeded along microcyst development without further delay. A series of 5-ml cell suspensions was prepared, and glycerol was added to each at time zero. At 25 min, 45 ml of 1% Casitone-0.01 M $MgCl_2$ was added. This 10-fold dilution of glycerol concentration effectively arrested all subsequent microcyst development. Cell suspensions were then incubated for serial times before the glycerol concentration was again restored to 0.5 M. For each suspension, the time required for the first appearance of cell shortening was determined by microscopic observations. These times were corrected for a control suspension to which 45 ml of 1% Casitone-0.01 M $MgCl_2$ containing 0.5 M glycerol had been added at 25 min.

When these corrected lag times were plotted against the time the cells had been incubated in the absence of an effective glycerol concentration, the curve shown in Fig. 3 was obtained. It is apparent that withdrawal of the inducer produced a progressive increase in the subsequent lag upon re-addition of the inducer, and hence a progressive reversal of the events occurring during the lag period. The whole of this lag period did not appear to be reversible during the time of these ex-

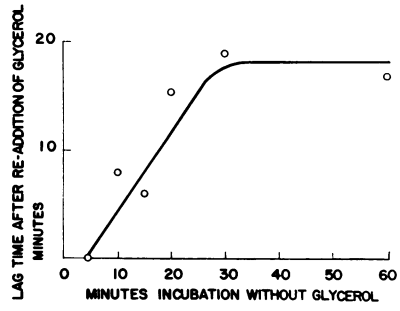

FIG. 3. *Effect of removal of glycerol on length of lag period.*

periments, as it never attained the full 25 min of the first incubation but leveled off at about 20 min.

DISCUSSION

Microcysts of *M. xanthus* induced in liquid medium by the addition of 0.5 M glycerol have been shown to possess the same characteristics as those produced in fruiting bodies on solid media (2). This system thus affords an opportunity for the investigation of the biochemical basis of a morphogenetic change in a simple system under controlled conditions.

As a preliminary to this investigation, we have first examined the overall pattern of macromolecular synthesis during the conversion process. The RNA, DNA, and protein contents of the microcyst all show increases of between 20 and 35% compared with their values in the vegetative cell. These increases occur in a two-step manner, there being a break in the rate of synthesis in all cases from about the time of initiation of cell shortening until the ovoid stage. This discontinuity in the rates of synthesis was not observed in studies on the incorporation of labeled uracil and amino acids into acid-insoluble material. This difference may reflect alterations in the rate and pattern of the turnover of macromolecular constituents during this period. In any case, it will now be of interest to characterize the macromolecular components synthesized during microcyst formation and to compare them with those from purely vegetative cells, and in particular to see whether microcyst-specific RNA and protein fractions are synthesized upon addition of glycerol. The fact that probably all stages of the conversion process are sensitive to both chloramphenicol and actinomycin D strongly suggests that the observed syntheses of RNA and protein are obligatory for microcyst development—if they are inhibited, no further development takes place.

In addition to glycerol, a number of other compounds have been found to effect microcyst conversion. All these compounds contain an alcohol group. The most effective inducers, i.e., those in the first category in Table 1, are fully saturated aliphatic compounds containing two to four carbon atoms and at least one primary or secondary alcohol group. In the homologous series of primary aliphatic alcohols, there is an optimal chain length for effectiveness, and, furthermore, the effective concentration for induction decreases with increasing chain length.

A number of experiments indicate that the mode of action of glycerol and erythritol may be identical. In investigating the question of the mechanism of inducer action, it was shown that the amount of glycerol incorporated by cells during their conversion to microcysts was only a very small fraction of the initial glycerol concentration. The observed rigid requirement for the high initial external concentration could then have either of two explanations. (i) It is required to force the entry of a small amount of glycerol which then induces microcyst development. (ii) The incorporated glycerol is irrelevant and it is the external glycerol which is effective.

Among other inducers, it was shown that the incorporation rates of isopropanol and erythritol were extremely low, and in the case of ethylene glycol cells appear to be totally impermeable to this compound. These data suggest that microcyst conversion may be brought about by an interaction between inducer and some peripheral structure of the vegetative cell. This is consistent with the observation that, after the addition of inducer, there is a sharp decrease in the optical density of the cells prior to any visible morphological change.

The fact that high concentrations of nonpenetrating solutes such as glucose do not induce microcyst formation suggests that induction is not the result of a simple osmotic effect. Since the presence of glycerol is continuously required, it appears that it is not acting as a simple trigger but is required to maintain a continuous altered state of the cell structure or organization. Furthermore, the sequence of events initiated by the addition of glycerol is largely reversed in a progressive manner after withdrawal of the inducer. This suggests that, although protein synthesis may be required during the lag period, an additional reversible change also takes place. It is interesting to note in this connection that it has been recently suggested that the site of action of phenethyl alcohol in inhibiting DNA synthesis in *Escherichia coli* may be the bacterial membrane (7). An association between DNA and the cell membrane of *Bacillus subtilis* has already been demonstrated morphologically (6) as well as biochemically (3). It is possible that the inducers of microcyst formation may alter a membrane-DNA relationship, initiating the read-off of that part of the genome associated with microcyst formation.

ACKNOWLEDGMENTS

We acknowledge the very capable technical assistance of Theresa Young and Sara Zeece.

This investigation was supported by grant GB-9 of the Development Biology program of the National Science Foundation. The junior author is a Public Health Service Career Development awardee (1-K3-GM-5869-10).

LITERATURE CITED

1. DWORKIN, M., AND S. M. GIBSON. 1964. A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **146**:243–244.
2. DWORKIN, M., AND W. SADLER. 1966. Induction of cellular morphogenesis in *Myxococcus xanthus:* I. General description. J. Bacteriol. **91**: 1516–1519.
3. GANESAN, A. T., AND J. LEDERBERG. 1965. A cell-membrane bound fraction of bacterial DNA. Biochem. Biophys. Res. Commun. **18**:824–835.
4. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
5. MANS, R. J., AND G. D. NOVELLI. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. **94**:48–53.
6. RYTER, A., AND F. JACOB. 1964. Étude au microscope électronique de la liaison entre noyau et mésosome chez *Bacillus subtilis*. Ann. Inst. Pasteur **107**:384–400.
7. TREICK, R. W., AND W. A. KONETZKA. 1964. Physiological state of *Escherichia coli* and the inhibition of deoxyribonucleic acid synthesis by phenethyl alcohol. J. Bacteriol. **88**:1580–1584.